CALCIFICATION IN MARINE MOLLUSCS *

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Introduction

The correlation between mantle activity and the production of shells in molluscs has been of interest for a considerable time. Studies by Rawitz (1888, 1890) have shown in considerable detail the histological structure of the mantle tissues of a number of species. Biederman (1914) and Schmidt (1923) described the morphological aspects of shell formation with particular reference to the pattern and arrangement of the various components. The reports of Moynier de Villepoix (1892), Brooks (1905), Hass (1935), Bourne (1943) and others too numerous to mention have also added considerably to our understanding of the structure, composition, crystalline pattern and in some degree to the detailed mechanism involved in the calcification of the shell.

Despite a voluminous literature (see Hass, 1935) which has appeared in connection with calcification in molluscs, several phases of this problem still await further study and clarification. By means of methods to be described in the following paragraphs we have examined in detail a number of aspects of shell formation and in so doing have utilized several different methods of attack upon the problem.

Material and Methods

The species of molluscs used in this investigation consisted of *Atrina rigida* (*Pinna*), *Pedalion alatum*, *Codokia orbicularis, Pinctata radiata*, and *Venus mercenaria*. For studies in a closed system the young spat of *Pedalion* proved to be most successful. For rapid regeneration studies in which a glass cover slip was placed in the mantle cavity (Brooks, 1905) *Pinna* proved to be efficacious. *Codokia* and *Venus* were better suited for histological and histochemical studies than other forms examined due to the relative lack of pigment in the mantle tissues.

Standard techniques and procedures were used for histological studies. Mucin was localized by Hoyer's thionin method, phosphatase, pre-formed phosphate and reticulum according to the methods of Gomori (1937, 1941), and calcium according to the method of Feigl (1943). The artificial sea water media used in our experiments was made up according to the formula of McClendon (Rogers, 1927).

In this study we were concerned with various factors relating to calcification. It was therefore necessary to choose a number of different test animals in order that different aspects of the problem could be investigated. For certain phases of the study, histological and histochemical observations were made, for others a change in the environment of the animal was initiated, in a third set of observa-

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tions calcification was induced on a glass cover slip (Brooks, 1905), while still another set of observations were obtained by means of time-lapse photography.[1]

In order to stimulate growth of the shells in several species, a portion of the shell was removed. By means of this procedure, we were able to obtain rapid regeneration and observe a number of details related to shell deposition in a relatively short time.

Observations

For an understanding of the processes which will be described later it seems appropriate at this juncture to indicate briefly the salient morphological features of the mantle of the mollusc. Essentially, the mantle consists proximally of a thin sheet of tissue which is applied to the inner surface of the shell, while distally the mantle breaks up into a number of tentacle-like folds usually three in number. During the process of shell formation these folds undulate back and forth in the region of the free margin of the shell. The greater part of the mantle is covered by epithelium which varies in character from low cuboidal to the high columnar form. Mucous glands are very numerous, widely distributed and contain granules which give a positive test for calcium phosphate.

In some respects the formation of the shell is reminiscent of the early histogenesis of membrane bone. There are two distinct processes which occur: (1) the elaboration of a fibrous organic membrane, and (2) the concentration and deposition of mineral salts.

The elaboration of the organic matrix is brought about by the activity of a layer of cuboidal cells occupying the outer surface of the middle fold of the mantle (Figs. 1 and 3). The secretion elaborated by these cells is known as conchin or periostrachum. This substance, first deposited as a thin sheet, comes to be arranged in prisms and striae in later development (Fig. 6) and according to our own and other observations (Hass, 1935) is a protein and contains one or more reducing sugars. When first elaborated it takes a positive reticulum stain, later it undergoes certain modifications in staining reactions; the prismatic portion is acidophilic, the inner portion exhibits basophilic properties.

The various steps in the process of calcification of the shell were studied advantageously by means of the "cover-glass" technique. As shown in Figures 7 and 8, the first observable indication of mineralization consists of a deposit of numerous minute granules which appear to be embedded in the surface of the conchin layer. According to our observations these granules consist of calcium phosphate, and preliminary tests[2] reveal the presence of both inorganic and organic forms.

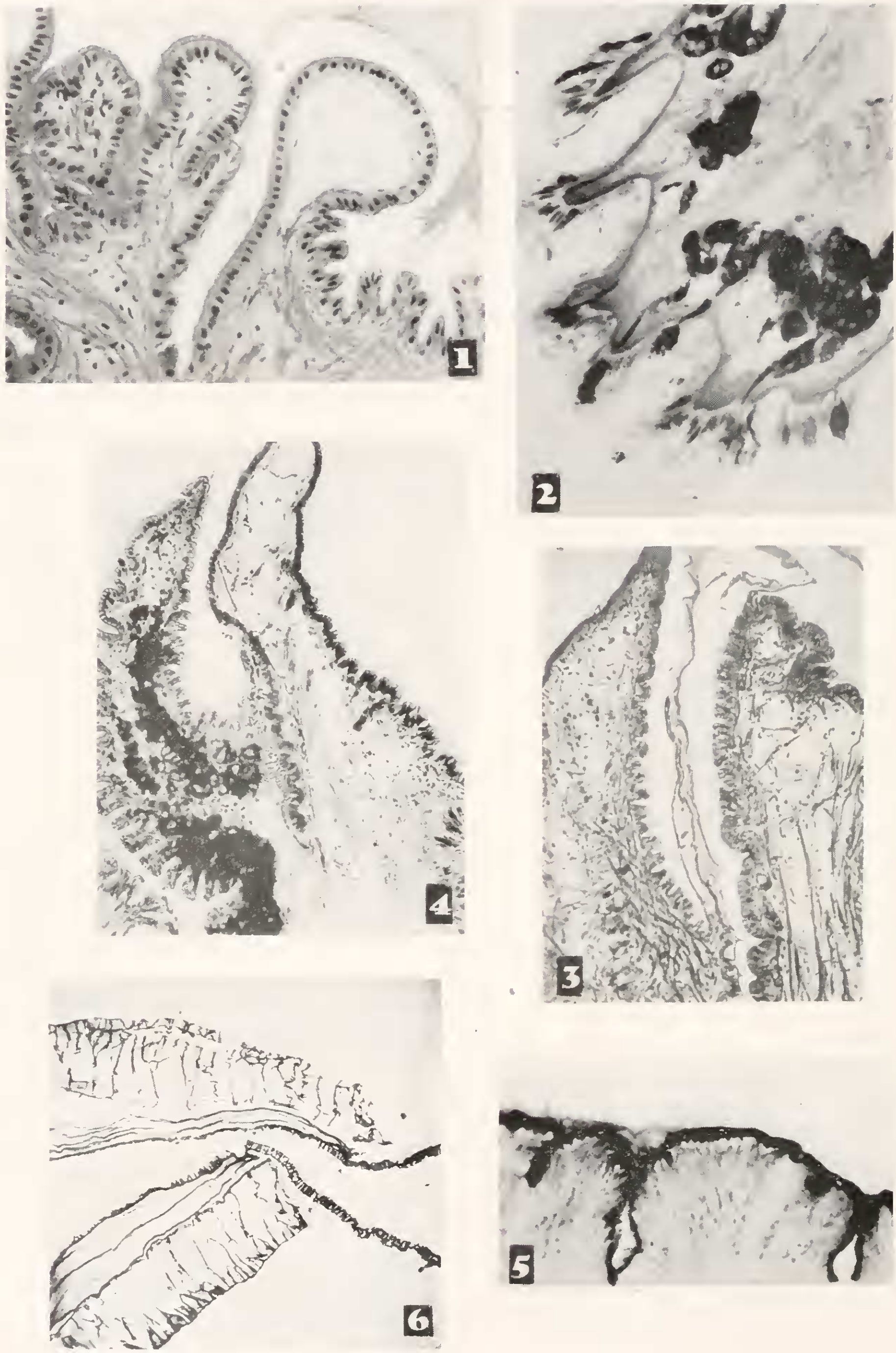
If one recalls that the mucous tissue is extremely abundant and widely distributed in the mantle tissues and that the mucous is secreted at the surfaces of the epithelial cells (Fig. 2), it becomes readily understandable that when the tentacles of the mantle undulate back and forth on the newly formed conchin layer, the granules will naturally be deposited on its surface and adhere thereto.

Once the granules are deposited in the conchin layer, they begin to undergo a typical crystal growth such as is shown in Figures 9 and 10. Continued growth

[1] Courtesy Mr. Edward Baylor, Princeton University.

[2] Tests made by Miss Lowell Lowell.

PLATE I



eventually results in an arrangement whereby the crystals (in *Pinna*) come to be enclosed in a thin layer of organic matrix and assume a polyhedral shape (Fig. 11). These crystals are composed of calcite (Biederman, 1914; Hass, 1935).

One interesting cytochemical feature which was observed in this connection was the identification of a strip of epithelium covering the surface of the inner middle fold which contains heavy concentrations of the enzyme alkaline phosphatase (Fig. 5).

Effect of Modifying Sea Water

Although lack of time did not permit a detailed study of the effect on calcification due to changes in the constituents of sea water, it was nevertheless possible to record a few pertinent observations in regard to this matter.

Using McClendon's formula for artificial sea water, young specimens of normal Pedalion were placed in this media; it was observed that they were alive and in good condition after a period of several days. Following this a number of specimens which had the free edge of the shell cut back as far as the mantle tissues were placed in this same media. Figure 12 is a photograph showing normal shell growth (regeneration) in a typical specimen.

Having shown that apparently normal calcification takes place under the conditions of the experiment, calcium was withdrawn in varying amounts from the artificial sea water and the resultant effects were observed in several specimens. The results of the experiments in which calcium was partially removed from sea water are shown in Figures 14, 15. Figure 12 shows a normal regenerating shell grown in sea water with the usual amount of calcium present. Figure 14 shows a specimen in which 50 per cent of the calcium was withdrawn, while Figure 15 illustrates a specimen grown in sea water containing only one-eighth of the normal calcium content. This latter specimen is completely devoid of calcium in the newly formed shell. Interestingly enough, however, the organic matrix was deposited in an apparently normal manner.

A few experiments were also made in which the magnesium content of the sea water was modified. Reference to Figure 13 shows that a similar condition may obtain when this mineral is withdrawn.

Discussion

In our studies concerning the calcification of the shell in several marine molluscs we have reviewed the essential histological structure of the mantle tissues and in so doing find that we are in all important respects in agreement with the reports of

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Figure 1. Longitudinal section of mantle of *Codokia*, hematoxylin and eosin showing formation of conchin.

Figure 2. Section of mantle of *Codokia* stained with thionin to show secretion of mucous.

Figure 3. Section of mantle of *Venus*, hematoxylin and eosin. General topography of folds and elaboration of conchin.

Figure 4. Mantle of *Venus* to show distribution of mucous. (Thionin)

Figure 5. Mantle of *Venus* showing distribution of alkaline phosphatase.

Figure 6. Decalcified section of shell of *Pedalion* showing arrangement of organic part of shell. Strand of tissue on right is regenerating tissue.

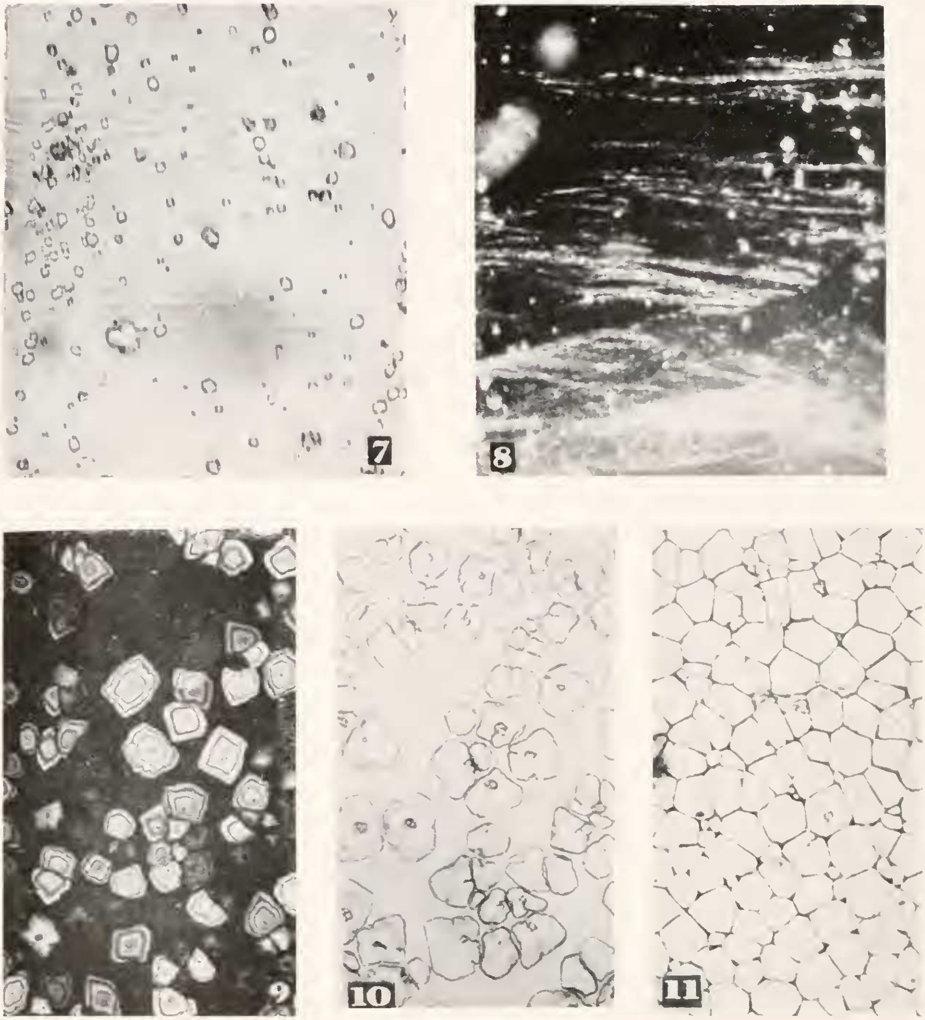

PLATE II

FIGURE 7. First formed elements of shell of *Pinna* deposited upon a cover slip. Note granules and fibrous matrix. Photo by transmitted light.

FIGURE 8. Similar preparation photographed by means of oblique illumination.

FIGURE 9. Crystal growth on cover slip preparation (*Pinna*). Photographed through crossed Nicols prisms.

FIGURE 10. Similar preparation photographed by means of transmitted light.

FIGURE 11. Mature shell (*Pinna*) surface view.

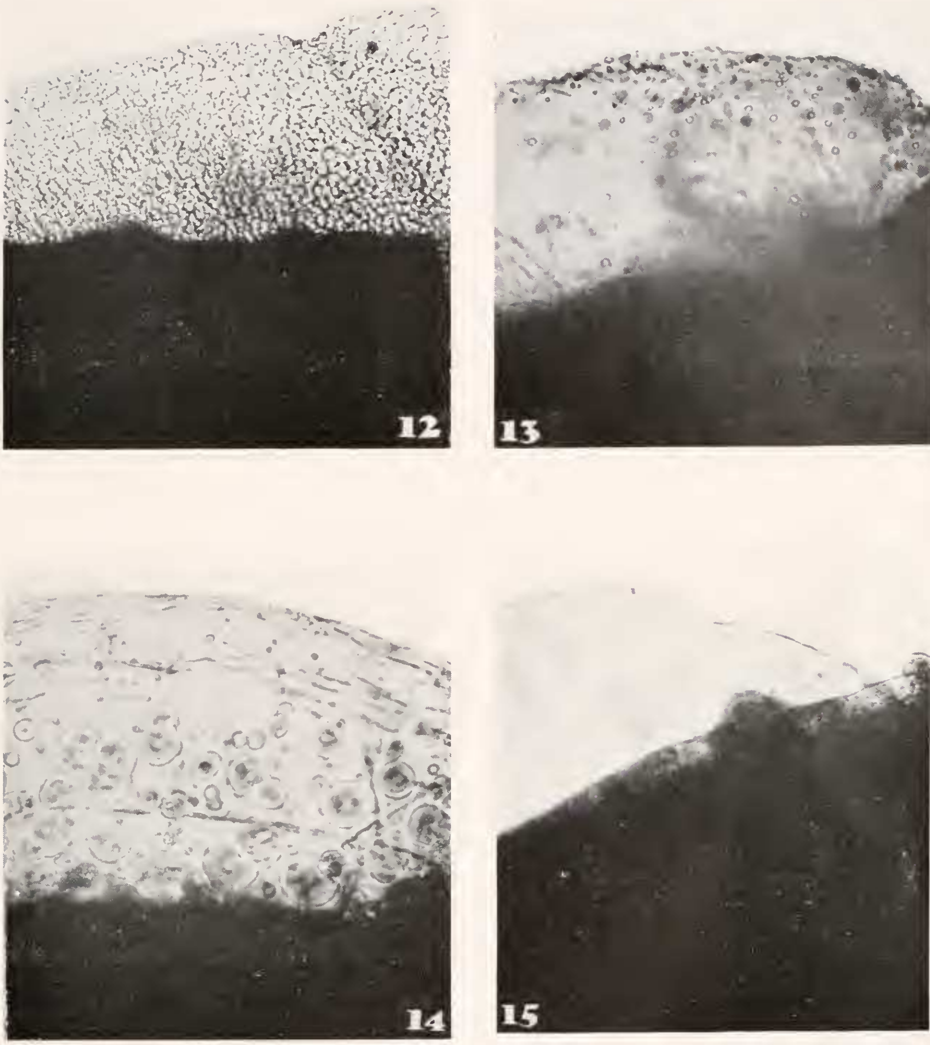

PLATE III

FIGURE 12. Normal regeneration of shell of young *Pedalion* in artificial sea-water.
FIGURE 13. Regeneration of *Pedalion* shell in which Mg was reduced.
FIGURE 14. Regenerating shell in 50 per cent normal Ca content.
FIGURE 15. Regenerating shell in one-eighth normal Ca content.

the earlier investigations (Rawitz, 1888, 1890). The observations of Biederman (1914), Schmidt (1923) and others in reference to the morphological aspects of shell formation have also been confirmed in part by our own observations.

In regard to the more minute details which occur during the process of calcification, Biederman (1914) refers to several pertinent observations and reports that while mantle tissue contains calcium phosphate in abundance, the calcified shell is made up of calcium carbonate and is completely lacking in phosphate. A similar statement appears in Plate (1922). Biederman further poses the interesting question concerning the mechanism of the transfer of phosphate to carbonate. Upon this point Moynier de Villepoix (1892) made some conjectures which seem to us at this time to have been extremely discerning: he states that it might be permissible to suppose that after the deposition of the granules in the conchin, that they might serve as the center of attraction of the elements and that the crystal growth of the calcium carbonate be determined in some manner thereby.

In regard to the presence of phosphatase on the surface epithelium of the mantle tissue, this condition was reported by Bourne (1943). Although this author's description is in many respects in agreement with our own observations, we are not certain that his photograph in fact indicates the presence of phosphatase, for our observations reveal that phosphatase is present on the surface of the cells which come in contact with the matrix and the granules, and not deeply embedded in the cytoplasm as indicated by Bourne.

In spite of the fact that many details have been omitted in this description of the process of calcification in the mollusc shell, it seems nevertheless feasible to suggest, if only in outline form, the essential steps which are involved:

An organic matrix is elaborated by epithelial cells located on the surface of the middle mantle fold. This substance gives a positive test for protein and reducing sugars. It also undergoes certain changes in arrangement and tinctorial properties as it matures.

The first observable anlage of the mineral part of the shell arises in the mucous glands, it is composed of calcium phosphate and comes to be deposited upon the matrix (conchin) due to the surface contact of the folds which pass over this tissue when the shell is being formed. The final calcified product is composed of calcium carbonate (calcite).

How this calcium phosphate is converted to the final calcified product, calcium carbonate, can be only conjectured at this time.[3] It does not seem likely that the conversion is a direct reaction between calcium phosphate and the carbonate of sea water for this process would require exceedingly high concentrations of carbonate.

Greenwald (1938) has emphasized the role played by other ions, particularly organic ones, in increasing the solubility of calcium phosphate. Such ions presumably act by binding calcium in some manner, possibly in an un-ionized moiety of the molecule. It is therefore proposed that the calcium phosphate may be dissolved by the participation of some organic ion. Phosphatase may aid this process by transferring phosphate to some substrate, thus removing the phosphate ions which otherwise would tend to slow the formation of the calcium complex. In a

[3] Freeman and Wilber (1948) have demonstrated the presence of carbonic anhydrase in mantle tissue and body fluids of some pelecypods and gastropods. However, these authors carefully point out that while it may have importance in shell formation in some species, negligible activity in others indicates that shell may be deposited in its absence.

further phase of this process, calcium ions may be made available by the alteration of the organic ion possibly through an oxidation-reduction reaction. If high local concentrations of calcium ions were thus made available, the carbonate of the medium would slowly precipitate calcium carbonate resulting in the crystalline architecture we have noted in our investigations.

Summary

1. Various aspects of the process of calcification have been studied in several marine molluscs.

2. Calcification of the shell is brought about by the formation of an organic matrix upon which minute granules of calcium phosphate are deposited.

3. In the presence of, and in contact with the mantle epithelium, crystal growth occurs in sea water.

4. The enzyme alkaline phosphatase appears to be concerned with calcification in these forms.

5. Modification of certain constituents of sea water results in the production of a shell partially or completely lacking in mineral content.

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